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Whole Exome Sequencing Identifies a Rare Mutation in *NACAD* as a Possible Cause of COVID Orchitis in Brothers

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Abstract

COVID orchitis (testicular pain) is reported in 10-15% of men with long COVID. We identified two siblings with COVID orchitis and hypothesized that genetic mutations are associated with susceptibility. Blood samples from five COVID-19 (+) men, three of whom had orchitis were evaluated by whole-exome-sequencing. A rare deletion on chromosome 7 was found in *NACAD* among the three men with orchitis. Interestingly, circulating ACE2 levels was decreased in men with COVID orchitis. This pilot study generated the hypothesis that men who develop COVID orchitis could have underlying genetic variants and altered levels in circulating ACE2 that may increase their risk.

Key Words

COVID-19, orchitis, genetics, ACE2, WES

Introduction

Since SARS-CoV-2 was declared a pandemic, efforts have mainly focused on mitigating primary infection mortality. During prioritization of community response and vaccine production, post-acute COVID-19 syndromes silently grew in prevalence¹⁻³. Organ inflammation syndromes are now well observed and have been documented in the heart, liver, kidneys, and testes⁴. While the blood-testis barrier is known to protect testes from antigens, current evidence suggests COVID-19 can affect the testis, either by (1) hematogenous spread due to periods of heightened viral load⁵ or (2) the host's immune response leading to proinflammatory cytokine release^{6,7}. With a similar mechanism to what was seen in SARS-CoV in 2002, SARS-CoV2 binds to the ACE2 receptor to be internalized⁸. This is a common receptor found in the serum that interestingly exists in the testes in high concentrations, and when viral bound, leads to viral internalization^{9,10}. As COVID-19 sequela outside of pulmonary symptomology continues to grow in prevalence, causal mechanisms need to be uncovered. Particularly, in cases of COVID-19 orchitis, a genetic mechanism is very likely due to a predilection for symptom precipitation in families^{11,12}. We hypothesized that a genetic mutation may be present in individuals who developed COVID-19 orchitis that causes increased viral entry and susceptibility to orchitis. Therefore, we performed whole exome sequencing on five individuals, two of which were brothers with COVID-19 orchitis and evaluated an association in serum circulating ACE2 levels.

Materials and Methods

Patient Selection

Patients were included based on COVID-19 (+) history and presence of or lack thereof orchitis symptomology (testicular pain). We identified six COVID-19 patients who all were confirmed to be COVID positive with polymerase chain reaction (PCR) tests. Patients included three COVID-

19 (+) men without orchitis (controls) and three COVID (+) men with orchitis (bilateral testicular pain for at least 5 days around the time of testing PCR positive). Among the three men with COVID-19 orchitis symptomology, two of them were siblings. All patients were identified at University of Miami School of Medicine Urology Clinics and provided informed consent for participation to be in accordance with the Declaration of Helsinki¹³. The study also received IRB approval (#20150740).

Whole Exome Sequencing

Peripheral venous blood samples were collected from each of the patients. DNA extraction was performed on blood using the QIAmp blood maxi kit (Qiagen, Germantown, MD) on five of the six patients. Whole exome sequencing was completed at John P. Hussman Institute. Captured exomes from DNA shearing were library prepared for amplification and sequencing. 16 variants were prioritized by being shared between the three patients affected with orchitis, absent in controls, and introducing nonsense, frameshift, splicing or non-synonymous amino acid changes and less than 10% in population prevalence.

Phenotypic Validation

DuoSet® Human ACE2 reagent kit 2 (catalog number: DY933-05) was purchased from R&D Systems, Minneapolis, USA, and enzyme-linked immunosorbent assay was used to measure duplicate levels of soluble ACE2 in the plasma samples.

Results

Case Report

Probands of this study were three patients who contracted COVID-19 and developed orchitis. All three subjects never experienced orchitis symptomology in the past and reported no previous family histories. The average age of the men in the study was 25 years old. They reported an

average duration of COVID-19 symptoms (fever, cough, and body aches) of seven days, and none required hospitalization. Among the men who developed bilateral testis pain, the symptoms lasted for an average of 22 days. The median sperm concentration and sperm motility was 19 million/ml and 60% around 3 months after original infection.

Whole Exome Sequencing Results

Whole exome sequencing yielded a total of 43,857 single nucleotide variants in at least one of the five participants. First, we filtered for variants shared between the two siblings with COVID orchitis along with the unrelated subject with COVID orchitis and for variants absent from the two controls, which returned 606 variants. Filtering for nonsynonymous mutations, including frameshift mutations, left 295 variants, which was reduced to 16 unique variants with a final filter of prevalence of 5% or less. Variants were individually analyzed and filtered based off criteria of (1) clear role in established COVID-19 linked orchitis ACE2 pathways and (2) read and genotype quality. Among the 16 candidate variants, we prioritized a nonsynonymous non-frameshift deletion in *NACAD* on chromosome 7: 45084258 with a frequency of 3.9% prevalence in ExAC, which causes a deletion of 120 base pairs. This variant was present in the three patients with COVID-19 orchitis and absent in the controls.

Phenotype Results

Phenotypically, we found median circulating levels of soluble ACE2 to be 3.40 ng/ml among men who had COVID orchitis. This was lower when compared to men who developed COVID without orchitis with median ACE2 levels of 4.33 ng/mL ($P < .05$, Wilcoxon signed-rank test). These ACE2 serum levels are consistent with existing evidence from COVID infected individuals¹⁴. As ACE2 serum levels normally range from 0.68 to 2.54 ng/mL in healthy

individuals¹⁵, baseline SARS-CoV2 infection is associated with elevated levels. This suggests that higher ACE2 serum levels may be protective from developing COVID-19 orchitis.

Discussion

We identified a unique patient group consisting of two brothers and one non-related individual who all experienced a rare sequela of COVID-19 infection. Both the siblings and the non-related patient possessed a nonsynonymous non-frameshift mutation in *NACAD*. Comprehensive search through whole exome sequencing studies regarding COVID-19 (+) severity showed no previous mention of this mutation¹⁶⁻¹⁸. *NACAD* gene products are thought to be involved with proper facilitation of polypeptide extracellular transport. Furthermore, the protein is thought to bind to nascent protein chains as translation occurs to facilitate proper interaction with the signal recognition particle occurs¹⁹. In tandem, impaired (1) polypeptide secretion and (2) endoplasmic reticulum targeting can lead to decreased extracellular protein secretion and increased cell membrane deposition of mistargeted proteins.

Non-frameshifting variants result in the gain or loss of several nucleotides divisible by three, such that the reading frame of the mRNA segment is not disrupted. The resulting mutant protein sequence differs from the wildtype with the addition and/or deletion of one or more amino acid residues. Phenotypic effects of a non-frameshift variant can result in impaired protein function and could impact biological pathways²⁰. Consequently, our findings suggest that the effect of the *NACAD* mutation results in modifying the extracellular and intracellular ACE2 concentrations as evident by the serology of ACE2 in COVID-19 orchitis patients compared to COVID-19 patients without orchitis. Particularly during peak viraemia, already elevated ACE2 membrane concentrations are faced with increased serum viral body concentrations to cause an increased risk of COVID-19 precipitated orchitis symptomology^{9,21}.

We hypothesized that disrupted extracellular transport and endoplasmic reticulum interaction leads to altered intracellular levels of ACE2. Subsequently, increased intracellular ACE2 levels result in cell membrane protein deposition. We identified a unique patient group with a rare COVID-19 sequelae, of mostly similar ages, none of which reported prior orchitis symptomology or family history. While we acknowledge limited subject verification, we have actively identified other families with similar patterns of orchitis sequelae and aim to study them. Ultimately, the combination of our genetic analysis and the difference in ACE2 levels between the groups suggests a possible mechanism for COVID-19 orchitis that warrants further investigation.

Conclusion

This is a pilot study that suggests a possible genetic link for increased risk of COVID-19 orchitis. We observed a mutation in *NACAD* in two brothers and one unrelated man who developed COVID-19 orchitis. Interestingly, we found lower circulating ACE2 serum levels in both brothers with orchitis and the one nonrelated orchitis subject but normal serum levels in all controls. Future studies focusing on evaluating the mechanism of how *NACAD* mutation will lead to changes in circulating serum ACE2 levels will uncover important mechanisms not just to understand why some men with COVID develop orchitis but also why some men develop multi-organ failure with COVID infection whereas most do not.

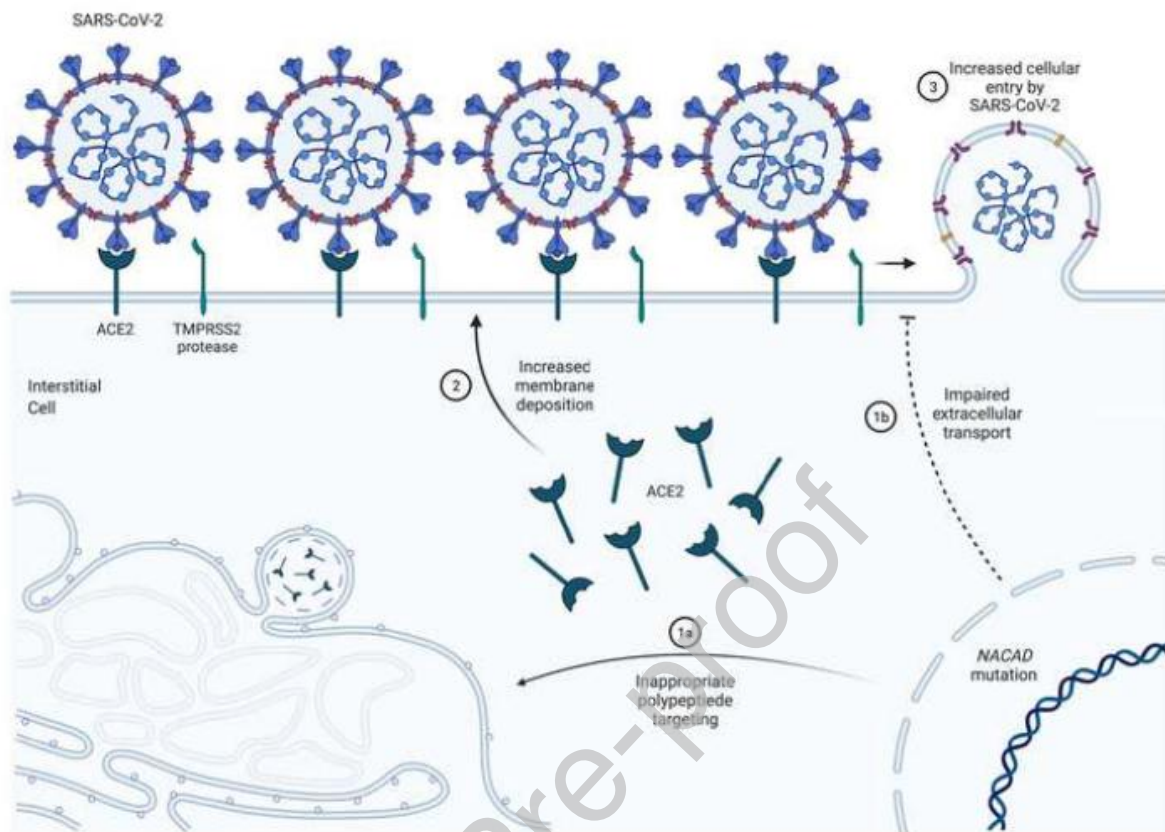


Figure 1. NACAD Pathway. The proposed role of NACAD and its interactions with polypeptide targeting via the endoplasmic reticulum (ER) and downstream changes in circulating ACE2.

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